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14. ABSTRACT

Following brain injury, there is an influx of inflammatory cells, especially macrophages, and there is reportedly widespread activation of microglia. Microglia have been divided into two major subgroups: (i) "classical" or "M1" macrophages, which promote inflammation and secrete IL-12, and (ii) alternatively activated or "M2" macriophages, which phagocytose apoptotic cells, promote wound repair, and (in mice) express arginase 1. We originally proposed that micriglial might also reflect these functional subsets whtih differential effects on TBI. To test this, we are studying TBI in "reporter" mice that express the fluor YFP undeer control of the promoter for either IL-12 or arginase 1. To date, we have not found activation of either IL-12, or arginase 1 in microglia following TBI, but we do find that TBI induces the CCR2-dependent influx of macrophages that express arginase-1, and that in the first day after injury about 20% of macrophages express arginase-1 at very high levels. We have not seen an effect of PPAR agonists on levels of this subset, but we have recently performed expression arrays on the post-TBI macrophages. The arrays demonstrate that these two cell populations differ from each other not only in the level of expression of arginase-1 but also in multiple other genes, especially chemokines. Neither cell population has the expression profile of M1or M2 cells. Instead, they represent novel macrophage cell populations. We are also performing microarray studies of microglia, following TBI. Our studies to date have not found evidence for microglial subsets, but by using a TBI model with greater impact than before, and by improving sensitivity of analysis, we do find that TBI induces widespread activation of microglia, as demonstrated by up-regulation of surface CD86.

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INTRODUCTION

Prior studies have provided evidence that traumatic brain injury may result in extensive activation of microglia, which phagocytose apoptotic neurons and initiate the process of brain repair (1). Activated microglia, however, can also initiate an inflammatory response, extending brain damage (1,2). Our studies seek to define if TBI induces subsets of microglia that may selectively protect against injury or worsen it. This hypothesis is suggested by studies of peripheral macrophages, which have demonstrated at least two major macrophage subtypes, called M1 and M2 (also called "alternatively activated" macrophages) (3,4). M1 macrophages are pro-inflammatory, while M2 macrophages are generally anti-inflammatory. M1 macrophages also promote insulin resistance and obesity, while M2 cells promote the action of insulin (5).

M1 cells express the p40 component of the IL-12 cytokine (IL12p40), while M2 cells are marked by the expression of arginase-1 (at least in mice). The activation of M2 macrophages is promoted by peroxisome proliferator-activated receptor γ (PPARγ), a nuclear receptor, and activation of PPARγ has anti-inflammatory effects both in the periphery and the brain (6-8). The related receptor PPARδ is also important in the activation of M2 cells (9). *In vitro* studies of microglia suggest that they, like macrophages, may be either proinflammatory or phagocytic, and that expression of the surface receptor TREM-2 promotes phagocytosis while suppressing the production of the inflammatory cytokine TNF-α (10).

Based on these findings, we have sought to test the central hypothesis that subtypes of microglia regulate the extent of TBI. To test this hypothesis, we have examined both microglia and macrophages in the mouse brain for the expression of markers that define M1 and M2 cells, and we have then tested the effect of TBI, using a controlled cortical impact model. We have also examined the effects of activating PPARs on these parameters. As will be discussed, the results of our studies have led us to focus more intensely on the macrophages that infiltrate the brain following TBI, but we will continue to examine both microglia and macrophages.

Our Specific Aims are:

1. Determine the development of microglia into M1 and M2 subtypes in response to TBI at multiple timepoints *in vivo* and establish the function of these microglial subtypes *ex vivo*.
2. Skew the microglial response to TBI towards an M2 phenotype using PPARγ and TREM-2 agonists as treatments given post-TBI.
3. Define the effects of PPARγ activation with and without and stimulation of TREM-2 on T cell infiltration and neuronal death in response to TBI.

BODY

Our primary approach to our analysis of microglial subsets is the use of reporter cell mice provided by our collaborator, Richard Locksley (UCSF). There are two strains: (i) yet40 mice, which express the fluor YFP under the control of the IL-12p40 promoter, a marker for M1 macrophages, and (ii) YARG mice, which express YFP under the control of the promoter for arginase-1, a marker for M2 macrophages.

The approved Statement of Work includes the following discussion of timelines:

Specific Aim 1. Determine the development of microglia into M1 and M2 subtypes in response to TBI at multiple timepoints *in vivo* and establish the function of these microglial subtypes *ex vivo.* As detailed in the application, these studies involve the isolation of microglia from mice at intervals after they are subjected to TBI and assessing their subtypes as determined in particular by flow cytometry to assess the activation of IL-12 or arginase-1, using the yet40 and YARG reporter mice for IL-12 and arginase-1, respectively. Additionally, the overall production of cytokines by microglia will be assessed by ELISA. Microglia will be assessed for the expression of TREM-2 and will be subjected to functional assays, including phagocytosis of neurons. We will conduct more experiments in YARG mice than in yet40 mice, because these will be the primary focus of subsequent therapeutic studies. These studies will be conducted in years 1 and 2. As detailed in the IACUC protocol, the estimated use of mice for experiments in year 1 is 110 yet40 mice and 280 YARG mice. In year 2, about half of the designated mice will be used for this specific aim, i.e., 55 yet40 and 140 YARG mice. At present, for all studies with mice we must breed twice the number of mice needed for experimentation, because there are no available studies to define whether we can mix the use of male and female mice in TBI. We will test this, but our current plan is to use only male mice, as these have been the usual focus of study of TBI. Specific Aim 1 will be completed in Year 2.

Specific Aim 2. Skew the microglial response to TBI towards an M2 phenotype using PPARγ and TREM2 agonists as treatments given post-TBI. These studies test the use of a PPARγ agonist to drive microglia to an M2 phenotype, as assessed by use of the yet40 and YARG reporter cell mice. Two dosing schedules will be tested, with examination following a schedule determined by Specific Aim 1. Next, mice will be treated both with the PPARγ agonist, to activate M2 microglia, and with antibody to TREM2, to test whether this will augment and/or sustain the M2 phenotype of microglia. Additionally, effects on the brain will be assessed by immunohistochemistry and staining for microglial activation and neuronal death. Work on this Aim will begin in year 2 and continue into year 3. In year 2, about half of the designated mice will be used for this specific aim, i.e., ~60 yet40 and 120 YARG mice. In year 3, yet40 mice will still be bred in case they are needed, but all experiments will be on YARG mice, including 60 for this Specific Aim, which will be completed in year 3.

Specific Aim 3. Define the effects of M2-like microglial activation by PPARγ and TREM2 on T cell infiltration and neuronal death in response to TBI. TBI invokes an invasion of T lymphocytes. This Specific Aim will test whether the activation of PPARγ and/or anti-

TREM2 will alter the influx of T cells. We will also correlate this with neuronal cell death, as assessed by TUNEL assay. These experiments will be entirely on YARG mice. They will begin in year 3, using 60 mice, and continue into year 4, using 120 mice. The Specific Aim will be completed in year 4.

Before discussing the results from the most recent (fourth) year of our grant, we will summarize the results from years 1, 2, and 3.

Year 1. We devoted most of year 1 to Specific Aim 1, developing the methods of TBI in our lab and examining macrophages and microglia with or without TBI for the expression of markers for M1 and M2 cells. Ultimately, we purchased our own machine for the delivery of controlled cortical impact, and we extensively modified this to improve the reliability of the procedure and to increase the rate of impact. By using the reporter cell mice we found little or no expression of IL12p40 or arginase-1 in resting microglia (identified by flow cytometry as expressing CD11b together with intermediate levels of CD45) or in brain macrophages (identified by flow cytometry as expressing CD11b together with high levels of CD45).

Year 2. In our second year, we focused heavily on the features of microglia and macrophages that are found in the brain following TBI. As detailed in prior reports, there were several interesting surprises. First, in assessing changes in microglia and macrophages following TBI, we found large changes in the number and features of macrophages, but few if any changes in the number and features of microglia. It was of course expected that microglia would not rapidly expand in number, but from the prior literature we expected that they would be activated by TBI. In our initial studies, however, we found little evidence that this is so; microglia showed no change in the expression of type II major histocompatibility antigens (MHCII) and with our initial protocols we saw no change in the expression of CD86. As will be discussed in the report for this year, however, our newer methods do reveal CD86 expression. TBI did not induce expression of YFP in microglia from either the yet40 or the YARG mice, indicating no activation toward either an M1 or an M2 phenotype.

In contrast to these findings with microglia, the expanded macrophage population was activated; many macrophages on the injured side of the brain expressed MHCII, and most expressed CD86. Further, a portion of these cells ($\leq$5%) from YARG mice expressed YFP at day 4 following injury, indicating that they are M2 macrophages (alternatively activated). Virtually all of the brain macrophages following TBI expressed the chemokine receptor CCR2, and this allowed us to demonstrate the macrophage response to TBI in mice genetically deficient in CCR2 was reduced by ~80%. This argues that the expanded macrophages seen in the brain following TBI are largely recruited from the periphery in a CCR2-dependent manner.

In our second year, we also initiated Specific Aim 2, to test the role of a PPARγ agonist in driving microglia to an M2 phenotype. At the suggestion of our collaborator, Ajay Chawla, we used rosiglitazone instead of 15-deoxy$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2), and we used two different protocols. We also added studies using an agonist of PPARδ, GW0742. This was based on studies in other laboratories, including studies by our collaborator Dr. Chawla, demonstrating that the induction of the M2 phenotype in macrophages is dependent not only on PPARγ but also on PPARδ (8). Using YARG mice to assess activation of arginase-1 as marker

for M2 cells, we did not find that rosiglitazone, either alone or in combination with GW0742, activated cells expressing arginase-1 above control levels in either microglia or macrophages at day 4 following TBI. These studies were confound, however, by the finding that DMSO alone, which is the vehicle for solubilizing rosiglitazone and GW0742, itself activates arginase-1 expression in macrophages and to a lesser extent in microglia (as assessed by YFP fluorescence). As will be discussed, in our third year we used a different approach, gastric gavage of drugs, to circumvent this problem, though again without an increase in YFP fluorescence.

Also in our second year, we examined the expression of TREM-2 on macrophages and microglia. In our proposed work, one approach to activating microglia toward an M2 phenotype was to have been the administration of monoclonal antibodies (mAb) to TREM-2. Despite studies from several labs, including our own, showing that TREM-2 is expressed in microglia within histologic sections from adult brain as well as on the surface of cultured neonatal microglia (11), we found find little or no TREM-2 expressed on the surface of freshly prepared microglia from adult brains, with or without TBI, and with or without DMSO or rosiglitazone. Therefore the use of anti-TREM-2 to activate M2 cells was not feasible. This led us to substitute studies with GW0742, as discussed above.

Year 3. Our third year of studies led to several important new findings, though first we had negative results again with rosiglitazone and GW0742. For these studies, to circumvent the problem that the solvent DMSO alone activates macrophages and some microglia when given intraperitoneally, we turned to the administration of rosiglitazone and GW0742 by gastric gavage. This was based on findings by our collaborator, Ajay Chawla, that the amounts of DMSO given by this route do not have systemic effects on macrophage activation. For these studies, the mice were given 250 μg of rosiglitazone and 250 μg of GW0742 (~10 mg/kg) in 100 μl of 0.5% methylcellulose by gavage daily beginning 4 days before TBI (including the day of TBI), and again 2 and 3 days after TBI. The mice were then sacrificed 4 days after TBI. Despite this improved approach, we saw no increase in arginase-1$^+$ cells compared to controls in 5 out 6 mice, although in one of the 6 the percentage of arginase-1$^+$ cells was notably increased.

While these studies were ongoing, we extended the time frame for our studies of the expression of both arginase-1 and IL-12 following TBI. Our prior studies had shown that the number of macrophages is higher at days 4 and 7 following TBI than at day 1 or after day 7, and that most of these macrophages are from the side of the brain ipsilateral to the injury, with much fewer on the contralateral side. We had therefore chosen day 4 for almost all of our studies of yet40 and YARG mice. When we now looked at day 1 and at day 7, we still found no expression of YFP in the yet40 mice, demonstrating that M1 cells are not common at any time during the week after TBI. In the YARG mice, however, we were surprised to find that YFP was expressed in many more macrophages at day 1 than at day 4; at day 1 about 20% of the ipsilateral macrophages expressed YFP above background, forming a distinct subpopulation of cells (Figure 1, next page). This was in contrast to $\leq$5% of cells at day 4. There was no expression of YFP in peripheral blood monocytes on either day 1 or day 4 (not shown). The YFP$^+$ cells and the YFP$^-$ cells expressed comparable levels of CCR2, suggesting that they are both dependent on CCR2 for their rise following TBI, though we cannot test this directly, because we do not have CCR2-deficient YARG mice. As shown in our prior reports, YFP$^+$ cells are still detectable at day 4

following TBI, but they are reduced to fewer than 5% of cells. We have since looked also at day 7, by which time they no longer form a detectable, distinct population.

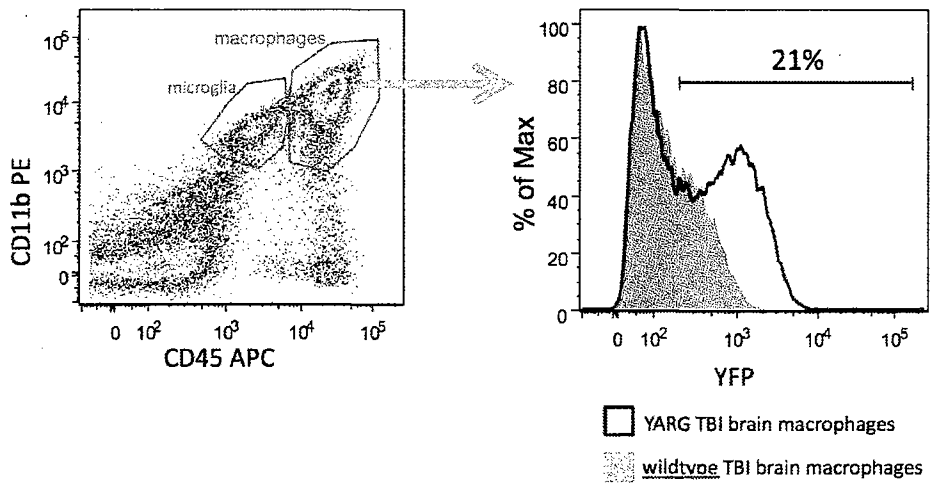

Figure 1. One day following TBI, ~20% of macrophages from the ipsilateral brain of YARG mice express YFP, indicating gene expression of arginase-1. Macrophages are identified by expression of CD11b and by high levels of expression of CD45. Their profile of YFP expression is shown in the right panel. Microglia also express CD11b, but express only intermediate levels of CD45. The microglia do not express YFP (not shown).

The expression of YFP by a substantial number of ipsilateral macrophages at day 1 following TBI made it possible for us to isolate these cells for further study. By combining ipsilateral brains from 4 YARG mice, and then separating YFP^+ macrophages from YFP^- macrophages by flow cytometry, we could obtain >20ng mRNA from each group, sufficient to produced an amplified cDNA array for study of gene expression. At the same time, we obtained peripheral blood monocytes from mice that been exposed to TBI and from those that had not. (We could not analyze macrophages from the brains of mice without TBI, because there are too few.)

The microarrays of brain macrophages subsets in TBI were successful. The analyses were largely done in year 4 and will be discussed there.

Year 4 (year of reporting)

<u>Microarray analysis confirms that at least two distinct subsets of macrophages are found in the brain following TBI</u>. Using brain cells obtained one day following TBI of YARG mice, microarrays from YFP^+ macrophages (hereafter called $Arg1^+$ macrophages) and YFP^- macrophages (hereafter called $Arg1^-$ macrophages) were compared to each other and to blood monocytes. In pairwise analyses of differentially expressed genes, both $Arg1^+$ and $Arg1^-$ brain macrophages showed numerous differences from normal blood monocytes, whereas monocytes from injured animals displayed few differences compared to normal monocytes (Figure 2A). Principal components analysis (PCA), an analytical technique that uses dimensionality reduction to identify patterns within data, confirmed that distinctions separating macrophages from

monocytes was the largest source of variance in the dataset (principal component 1), and that monocytes from injured or normal animals had fewer differences that were not represented in either of the top two principal components (Figure 2B). Scatter plot analysis of gene expression also revealed that a large number of genes (1,360 genes) differed significantly between $Arg1^+$ and $Arg1^-$ brain macrophages (Figure 2A). PCA demonstrated that $Arg1^+$ and $Arg1^-$ brain macrophages represented two distinct populations represented in the second most significant principal component (PC2) (Figure 2B).

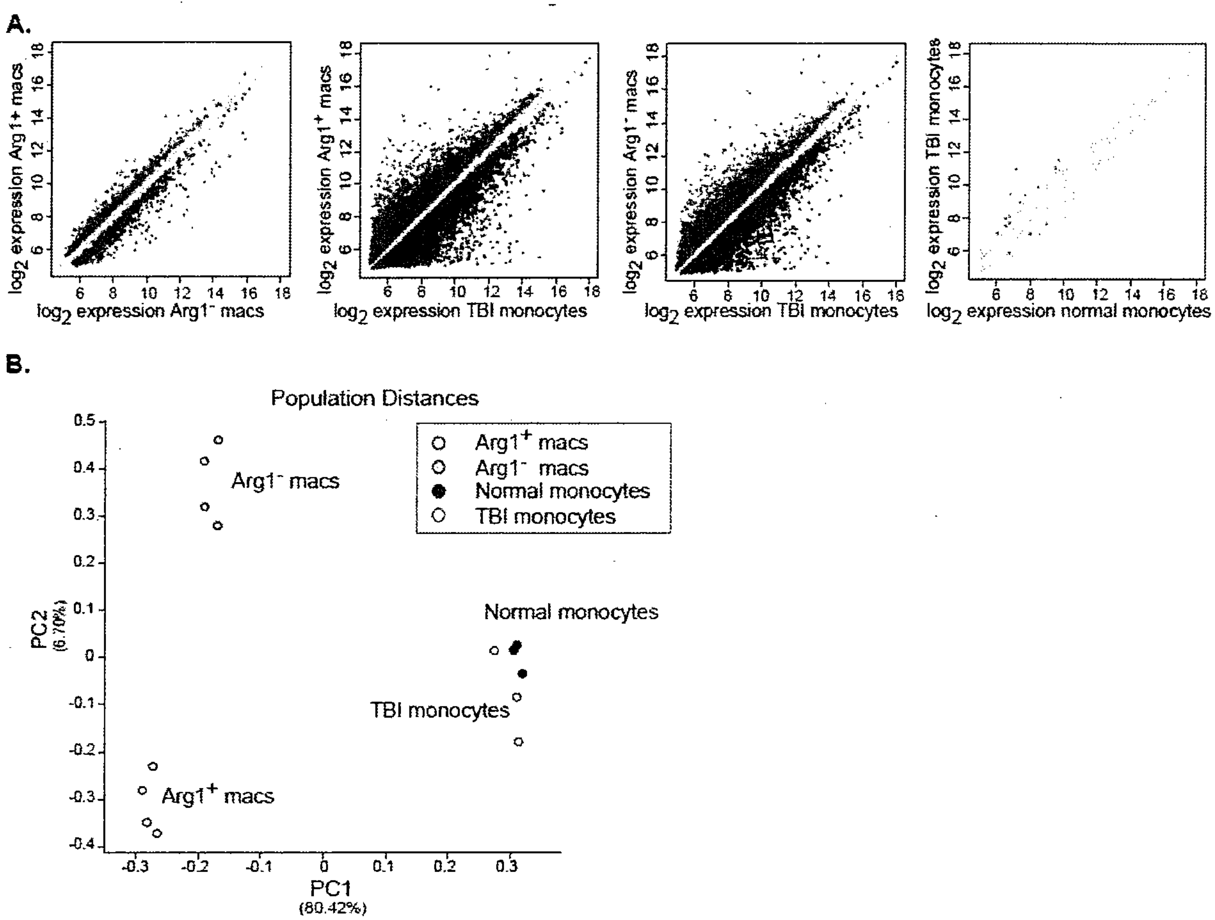

Figure 2: Gene expression profiling reveals extensive differences between $Arg1^+$ and $Arg1^-$ macrophage subsets.
A. Microarray data comparing gene expression by $Arg1^+$ TBI macrophages, $Arg1^-$ TBI macrophages, TBI monocytes, and normal monocytes were examined in a pairwise analysis. Red and blue color dots represent genes with significant differences. 1,360 genes differed between $Arg1^+$ macrophages and $Arg1^-$ macrophages; 11,799 genes differed between $Arg1^+$ macrophages and TBI monocytes; 9,932 genes differed between $Arg1^-$ macrophages and TBI monocytes; and 15 genes differed between the monocyte populations.

B. PCA of gene expression reveals large differences between monocytes and both Arg1$^+$ and Arg1$^-$ macrophages (PC1), and that Arg1$^+$ and Arg1$^-$ macrophages can be defined as distinct subsets (PC2).

The two subsets of macrophages do not represent M1 or M2 macrophages, but rather are unique cell populations. The expression of arginase-1 is a common marker for M2 macrophages, and our hypothesis was that the Arg1$^+$ macrophages would be M2 cells. The gene expression profiles of the Arg1$^+$ macrophages and the Arg1$^-$ macrophages, however, indicated that neither cell population represents M1 or M2 cells. Thus, the arrays confirmed that the Arg1$^+$ macrophages expressed high levels of arginase-1, and they also preferentially express another marker for M2 cells, *Mrc1*, encoding the mannose receptor/CD206. The Arg1$^+$ macrophages, however, also preferentially expressed *Nos2*, a gene associated with the M1 phenotype, not the M2 phenotype.

Similarly, although Arg1$^-$ macrophages had increased expression of *Il1b* (interleukin-1β), an inflammatory cytokine associated with M1 cells, they also preferentially expressed the signature M2 markers, *Retnla* (resistin-like α), and *Clec10a* (C-type lectin domain family 10, member A)/CD301 (Figure 3A and 3B, next page).

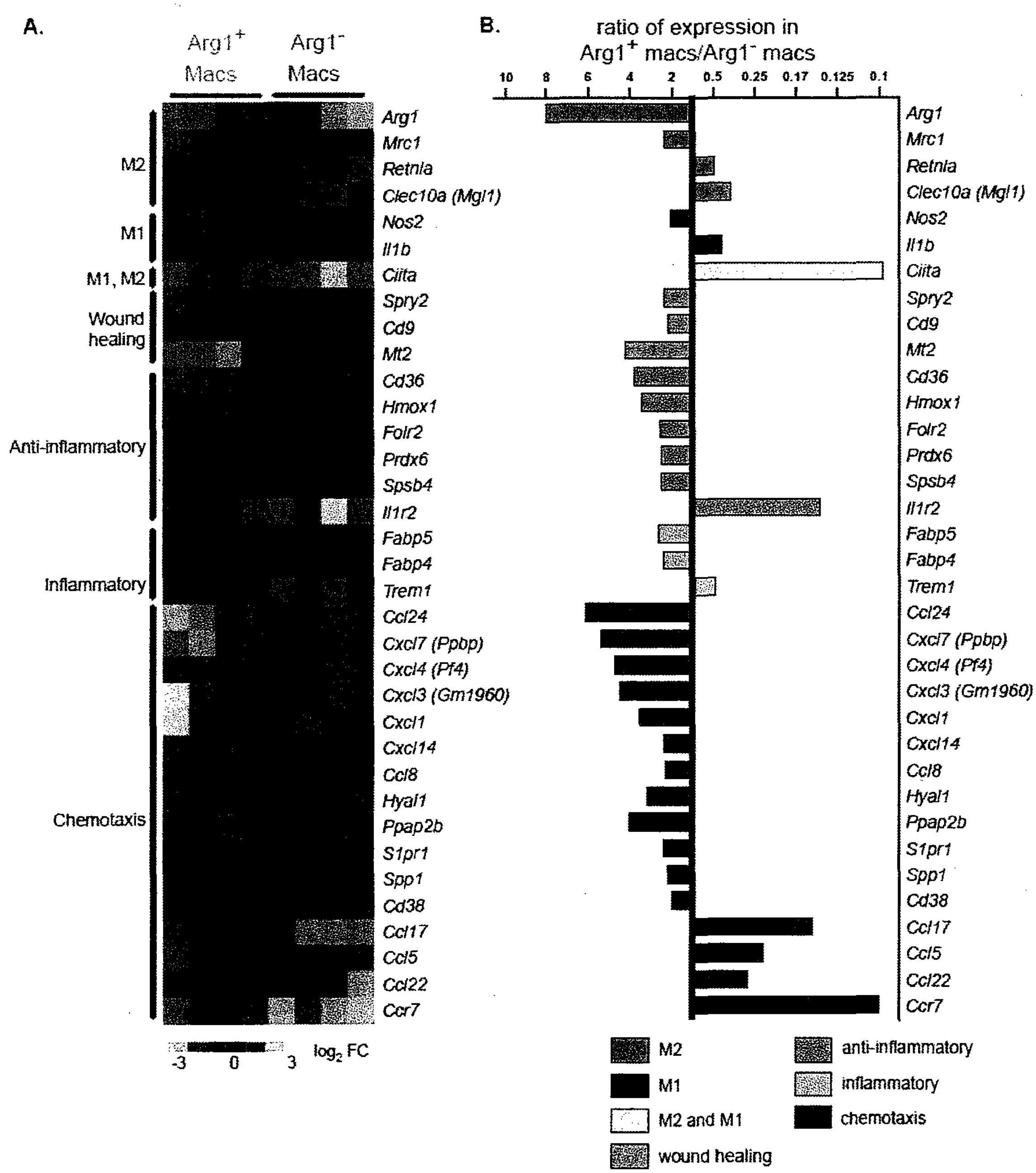

Figure 3: Comparison of transcriptional responses between Arg1$^+$ and Arg1$^-$ brain wound macrophages reveals profiles unique from M1 and M2 cells and possible functional roles.

A. Gene expression analysis comparing Arg1$^+$ TBI macrophages and Arg1$^-$ TBI macrophages one day after injury. Each column represents a replicate experiment (four for each cell population). Gene expression was $\log_2$ transformed and median-centered across genes. Yellow represents a relative increase in expression and blue signifies a relative decrease. Arg1$^+$ and Arg1$^-$ macrophages preferentially expressed unique patterns of genes.

B. Average gene expression differences were quantified and ratios of gene expression levels comparing Arg1^{+} brain wound macrophages to Arg1^{-} brain wound macrophages are shown. Arg1^{+} macrophages preferentially express transcripts for the M2 markers *Arg1* (8-fold) and *Mrc1* (2.4 fold), as indicated by bars to the left of the Y-axis, but Arg1^{-} brain wound macrophages preferentially expressed transcripts for the M2 markers *Retnla* (2.1 fold), and *Clec10a* (CD301/*Mgl1*) (2.9 fold) as indicated by bars to the right of the Y-axis. Arg1^{+} macrophages preferentially expressed the M1 gene, *Nos2* (2.1 fold), while Arg1^{-} expressed *Il1β* (2.4 fold) and *Ciita* (10 fold). Arg1^{+} macrophages had increased expression of *Spry2* (2.4 fold), *Cd9* (2.2 fold), and Mt2 (4.2 fold). Genes that have described anti-inflammatory function and that were relatively increased expression in Arg1^{+} cells include *Cd36* (3.8 fold), *Hmox1* (3.4 fold), *Folr2* (2.6 fold), *Prdx6* (2.5 fold), and *Spsb4* (2.5 fold), while *Il1r2* is more highly expressed (7.1 fold) in Arg1^{-} macrophages. The inflammatory genes, *Fabp5* and *Fabp4* were increased in Arg1^{+} macrophages, 2.6 fold and 2.4 fold respectively, while *Trem1* was increased in Arg1^{-} macrophages (2.1 fold). For chemotaxis associated genes: Arg1^{+} macrophages had higher levels of transcripts for *Ccl24* (6.2 fold), *Cxcl7/Ppbp*; 5.4 fold), *Cxcl4/Pf4*; 4.8 fold), *Cxcl3/Gm1960* (4.5 fold), *Cxcl1* (3.6 fold), *Cxcl14* (2.4 fold), *Ccl8* (2.3 fold), *Hyal1* (3.2 fold), *Ppap2b* (4 fold), *S1pr1* (2.4 fold), *Spp1* (2.2 fold), and *Cd38* (1.98 fold). Arg1^{-} brain wound macrophages showed increased levels of *Ccl17* (6.8 fold), *Ccl5* (4.4 fold), *Ccl22* (3.7 fold), and *Ccr7* (10 fold).

The transcript expression levels of *Arg1*, *Mrc1*, *Nos2*, and *Il1b* in TBI macrophages were confirmed by real-time PCR demonstrating that relative to GAPDH, these genes were indeed transcriptionally active (Figure 4). In accordance with flow cytometry data (Figure 5), gene expression analysis of MHCII, a molecule thought to be on both M1 and M2 cells, revealed that the Arg1^{-} macrophage population as a whole expressed much higher levels of MHCII transcripts (not shown) and higher levels of *Ciita* (class II, major histocompatibility complex, transactivator) than the Arg1^{+} macrophages (Figure 3A and 3B), suggesting functional differences in the capacity to present antigen to CD4^{+} T cells.

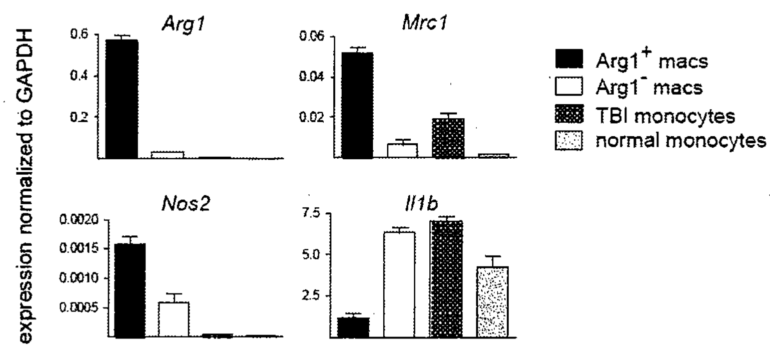

Figure 4: Real-time PCR data confirming expression of selected M2, M1 and chemotaxis associated genes corroborates microarray results. Arg1^{+} TBI brain macrophages expressed *Arg1, Mrc1,* and *Nos2*. Arg1^{-} TBI brain macrophages expressed relatively lower levels of these genes compared to Arg1^{+} TBI brain macrophages, but had increased expression of *Il1b*. Data represent mean ± SEM.

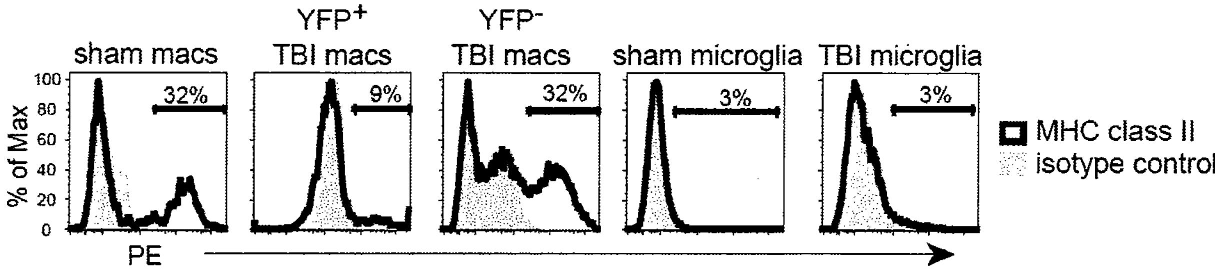

Figure 5: TBI induces a subset of YFP⁻ MHCII⁺ macrophages in the injured brains of YARG reporter mice.

YFP$^+$ and YFP$^-$ ipsilateral brain wound macrophages from YARG mice, and ipsilateral macrophages from sham controls were assessed by flow cytometry for the expression of MHCII. YFP$^+$ brain wound macrophages expressed little or no MHCII, while a subset of YFP$^-$ and a subset of sham brain macrophages expressed MHCII. Microglia did not express detectable MHCII.

We conclude that Arg1$^+$ macrophages and Arg1$^-$ macrophages have mixed expression of M2 and M1 properties. To further compare these cell populations to classic M1 and M2 cell populations, we performed a meta-analysis of significant genes differentially expressed between Arg1$^+$ and Arg1$^-$ TBI brain macrophages with significant genes differentially expressed between IFNγ-stimulated or IL4-stimulated bone marrow-derived macrophages (BMDM) (Zhang et al., 2010). Arg1$^+$ and Arg1$^-$ macrophages each upregulated genes that were induced by in BMDM by either IFNγ or IL-4 stimulated BMDM, supporting the notion that Arg1$^+$ and Arg1$^-$ TBI brain macrophage subsets have complex expression profiles with a mixed M1 and M2 phenotype (Figure 6, next page).

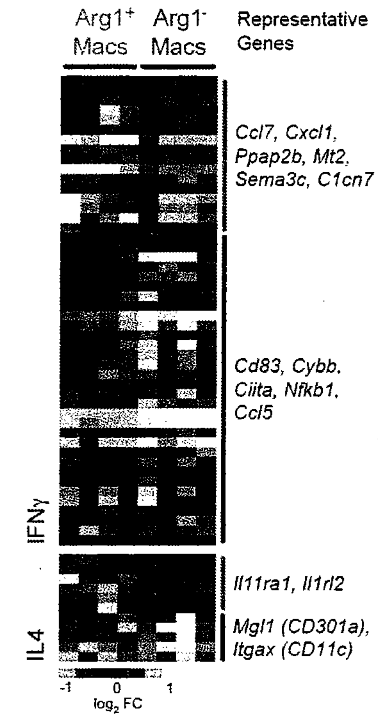

Figure 6: TBI macrophage subsets preferentially express different groups of genes induced by IFNγ or IL4 stimulation. Genes that were differentially expressed between IFNγ-stimulated and IL-4 stimulated bone marrow-derived macrophages were compared for their expression between Arg1$^+$ macrophages and Arg1$^-$ macrophages. The data were median-centered across genes. Each TBI brain macrophage subset preferentially expressed some genes that were stimulated by IFNγ and some genes that were induced by IL4. To the right, genes representing each cluster are shown.

<u>Arg1$^+$ macrophages and Arg1$^-$ macrophages in the brain following TBI differ notably in their expression of chemokines</u>. Although the Arg1$^+$ macrophages and Arg1$^-$ macrophages are neither M1 nor M2 cells, they are clearly distinct from each other. The most striking and perhaps most novel differences between Arg1$^+$ and Arg1$^-$ macrophages are in their unique chemokine profiles. Arg1$^+$ macrophages preferentially express a chemokine repertoire that includes *Ccl24* (eotaxin), Cxcl7 (pro-platelet basic protein, *Cxcl4* (platelet factor 4), *Cxcl3* (MIP2β, GRO3), *Cxcl1* (GROα), *Cxcl14*, and *Ccl8* (MCP-2; 2.3) (Figure 3A and 3B). Arg1$^-$ macrophages have their own chemokine expression pattern. They preferentially upregulate *Ccl17*, *Ccl5*, *Ccl22*, and *Ccr7* (Figure 3A and 3B).

In all, the gene expression signatures of Arg1$^+$ and Arg1$^-$ macrophages suggest the presence of functionally distinct TBI-responsive macrophage subsets with complex roles in promoting and suppressing inflammation as well as possible pleiotropic effects on CNS cells.

<u>Expression profiles of microglia following TBI.</u> In our studies of YARG and Yet40 mice, we have never seen expression of YFP in microglia from either strain either before or following TBI. Thus, if the expression of either arginase-1 or of IL-12p40 is increased in microglia

following TBI, it is not detectable by this approach. Initially, we also failed to see any change in surface markers on microglia following TBI. In studies over the last two years, however, we have seen widespread expression of CD86 on microglia following TBI, which is in accord with prior reports that there is widespread activation of microglia following TBI. We think it likely that we missed this initially because we started with a lower impact TBI, and because we have refined our use of multi-color flow cytometry so that it is more sensitive. The expression of CD86 is on almost all microglia, so this does not provide an approach to identifying microglial subsets. It does raise the important question, however, of what other microglial properties are altered by TBI.

To answer this question, we have prepared microglia from TBI and sham mice for analysis by microarray. Some of the samples from our initial experiments were inadequate, be we are repeated the experiments and expect to complete this analysis during the coming year of study (funded by a no-cost extension).

Presented below are the deliverables included in our approved Statement of Work, with notation on their status:

End of year 1

1. Quantification of microglial subsets in normal mice by use of reporter cell mouse lines yet40 and YARG. *Completed. Resting microglia do not express detectable transcripts for YFP in either reporter strain.*
2. Quantification of the expression of TREM2 on microglial subsets in normal mice as identified by use of reporter cell mouse lines yet40 and YARG. *Completed. Resting microglia from either strain or from wild type mice express little if any TREM-2 on the cell surface. By immunofluorescence microscopy, however, we and others have detected TREM2 within most microglia (by histology), leading to the conclusion that most TREM2 is inside the microglia rather than on the surface.*
3. Quantification of the production of the cytokine TNFα, as determined by ELISA, by microglial subset from normal mice, as identified by use of reporter cell mouse lines yet40 and YARG. *Largely completed, with some changes in approach. Our studies of yet40 mice could not detect a deviation toward an M1 phenotype (which is associated with the expression of TNFα) in either normal microglia or macrophages, and the ELISA proved insufficiently sensitive to detect amounts of TNFα that might be produced by the numbers of microglia obtained. We therefore turned to two more sensitive approaches. The first is expression arrays. In brain macrophages following TBI, both $Arg1^+$ and $Arg1^-$ cells expressed elevated levels of TNFα, compared to monocytes. Microarray studies of microglia are in progress. Second, by using PCR, we confirmed increased expression levels TNFα in brain macrophages following TBI. In microglia, the expression of TNFα appears to be even higher than in macrophages. This surprised us, and we await the results of the expression arrays of microglia to confirm this. These will be completed in the coming year.*

End of year 2

4. Quantification of microglial subsets following TBI by use of reporter cell mouse lines yet40 and YARG. *Completed. TBI alone does not induce detectable activation of YFP in the microglia from either yet40 or YARG mice. While this suggests that*

microglia do not differentiate toward an M1 or an M2 phenotype following TBI, our recent results with microarrays of brain macrophages indicate that the YARG mice may not be sufficiently sensitive to changes in arginase-1 that may nonetheless be significant. We are therefore performing microarrays of microglia both before and following TBI to re-examine this issue. Further, regardless of the results with microglia, our studies have shown that TBI induces a marked CCR2-dependent influx of macrophages. By analysis of YARG mice, a subset of these macrophages expresses arginase-1 at high levels, and by recent microarray studies, it may be that most of the macrophages express arginase-1, even if it cannot be detected in the YARG mice.

5. Quantification of TREM2 expression on microglial subsets following TBI, as identified by use of reporter cell mouse lines yet40 and YARG. *Completed. As with resting microglia (discussed above), freshly prepared microglia from mice subjected to TBI do not express detectable levels of TREM-2 on the cell surface. Because of these findings, we last year dropped the goal of using anti-TREM-2 to alter the function of microglia (and macrophages) but we added studies to assess the effect of the PPARδ agonist GW0742.*
6. Quantification of the expression of the cytokine TNFα as determined by ELISA, by microglial subset following TBI, as identified by use of reporter cell mouse lines yet40 and YARG. *Largely completed with necessary modifications. See #3 above, re studies in normal mice, which also discuss our results and plans in mice following TBI. The remaining studies (microglia arrays) will be completed in the coming year.*
7. Quantification of phagocytosis by microglial subset following TBI, as identified by use of reporter cell mouse lines yet40 and YARG. *These studies have not yet been possible because TBI does not induce detectable activation of microglia subsets. Last year, we proposed to pursue them if PPAR agonists could activate microglia. As discussed next, these studies proved to be negative. However, we now plan to perform expression arrays on microglia following TBI. Although this will not allow the detection of microglial subsets, we will examine phagocytosis by the microglia if the arrays suggest that the microglia are activated.*

End of year 3

8. In YARG mice, define the effects of the PPARγ agonist 15-deoxy$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2) on microglial activation and neuronal death *in vivo*, as assessed by flow cytometry and immunohistochemistry. *Studies of microglia completed. As advised by our collaborator, Ajay Chawla, we have used rosiglitazone as a PPARγ agonist instead of* 15d-PGJ_2. *Also, because activation of the M2 phenotype is now known to depend on PPARδ as well as PPARγ, we added studies testing of the PPARδ agonist GW0742, both alone and in combination with rosiglitazone. Even the combination of these agents, however, has not altered the profile of YFP expression in either microglia or macrophages from YARG mice, either before or after TBI.*

End of year 4

9. In YARG mice, define the effects of 15d-PGJ_2 with or without antibody to TREM2 (delivered intracranially) on the response to TBI, as assessed by T cell infiltration of the brain and neuronal death. *Altered and completed with regard to T cells. As discussed above, TREM-2 has not yet been detected on the surface of freshly prepared adult microglia with or without TBI, and this was not altered by the addition of PPARγ and PPARδ agonists. We therefore last year abandoned the use of*

anti-TREM-2, and substituted studies of the PPARδ agonist, GW0472. However, even the combination of the PPARγ agonist rosiglitazone and the GW0472 failed to alter the expression of YFP in YARG mice. Further, we could not detect changes in the T cell population. We therefore have not pursued studies of neuronal death following these agents.

KEY RESEARCH ACCOMPLISHMENTS

- As detected by the use of the reporter mice, yet40 and YARG, TBI alone in our model does not cause widespread activation of microglia nor expression markers for the M1 or M2 phenotype (IL12p40 or arginase-1, respectively).
- TBI, however, induces a large influx of macrophages.
- The influx of macrophages is largely dependent on CCR2, evidence that they are recruited from the periphery and providing a mean of studying the importance of these cells in TBI.
- As detected by expression of YFP in the YARG reporter mice, about 20% of the infiltrating macrophages express arginase-1 within a day following TBI. Although the number of macrophages is higher at days 4 and 7, the percent with detectable expression of YFP is highest at day 1, declines at day 4 and is not detected at day 7.
- Gene expression arrays confirm that the $Arg1^{+}$ and the $Arg1^{-}$ macrophages identified by YARG reporter cell mice differ in the expression of many genes, notably cytokines. Thus they represent distinct cell populations.
- Neither the $Arg1^{+}$ nor the $Arg1^{-}$ macrophage are M1 or M2 macrophages, as assessed by the gene expression profiles.
- As a direct result of this DoD work, the postdoctoral fellow who has pursued these studies (her first of TBI) was awarded a VA Career Development Award to pursue the importance of CCR2 in the functional consequences of TBI. This began in January, 2011. Further, she been appointed as an Assistant Researcher at UCSF and has been afforded PI status at the VA.

REPORTABLE OUTCOMES

- We have submitted our findings for publication, but they have not yet been accepted. We are revising the paper for submission to the Journal of Neuroinflammation. Dr. Hsieh presented her DoD-sponsored studies regarding the effects of TBI on macrophages as a poster at the 10^{th} Annual Meeting of the International Society of Neuroimmunology, Barcelona, October 26-30, 2010.

CONCLUSIONS

Our studies have shown that resting microglia do not express markers for an M1 or an M2 phenotype, as detected by reporter mice, nor are these markers induced by TBI. TBI, however, induces a marked influx of macrophages, which is dependent on CCR2, and as detected by YARG reporter mice, about 20% of these cells express arginase-1 at high levels one day after TBI, suggesting that they may be of M2 phenotype. Gene expression analysis of these cells, however, indicates that they are neither M2 nor M1 cells, but instead have a unique phenotype. They differ from the $Arg1^{-}$ cells in the expression of many genes, especially the expression of chemokines.

Initially, we did not detect activation of microglia following TBI as assessed by the expression of surface markers. With a larger impact and with improved FACS methodologies, we now detect CD86 on most microglia following TBI, consistent with widespread activation of microglia. We are now performing microarray studies on these cells to assess changes in activation.

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APPENDICES

List of Personnel. This was unchanged during the past year. Personnel include:

William E. Seaman, M.D. Principal Investigator
Mary C. Nakamura, M.D. Investigator
Christine L. Hsieh, Ph.D., Postdoctoral Fellow
Erene Niemi, Research Technician

SUPPORTING DATA

All figures and/or tables are imbedded in the report.